BRNO UNIVERSITY OF TECHNOLOGY
VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF ELECTRICAL ENGINEERING AND COMMUNICATION
FAKULTA ELEKTROTECHNIKY
A KOMUNIKAČNÍCH TECHNOLOGIÍ

DEPARTMENT OF BIOMEDICAL ENGINEERING
ÚSTAV BIOMEDICÍNSKÉHO INŽENÝRSTVÍ

SIMULATION OF ADHESION AND TRANSMIGRATION IMUNE-CELLS THROUGH CAPILLARY WALL
SIMULACE ADHEZE A TRANSMIGRACE IMUNITNÍCH BUNĚK STĚNOU KAPILÁR

BACHELOR'S THESIS
BAKALÁŘSKÁ PRÁCE

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VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ | FAKULTA ELEKTROTECHNIKY A KOMUNIKAČNÍCH TECHNOLOGIÍ

Bakalářská práce

bakalářský studijní obor **Biomedicínská technika a bioinformatika**

Ústav biomedicínského inženýrství

Studentka: Katsiarina Morgaenko ***ID:*** 164213

Ročník: 3 ***Akademický rok:*** 2015/16

NÁZEV TÉMATU:

Simulace adheze a transmigrace imunitních buněk stěnou kapilár

POKYNY PRO VYPRACOVÁNÍ:

1) Proveďte literární rešerši v oblasti in-vitro experimentů s endoteliálními buňkami ve 2D a 3D kultivačních zařízeních a v oblasti mikrofluidních systémů simulujících kapiláry. Prostudujte mikroskopické techniky pro snímání endoteliálních buněk. 2) Seznamte se s technikou konfokální mikroskopie. Navrhněte tvar a velikost vzorků s endoteliálními buňkami, která bude ideální pro dostupný mikroskop. 3) Navrhněte in-vitro kultivační jamky, kde je možno vytvořit vrstvu endoteliálních buněk a mikroskopem pozorovat prostor na jedné a druhé straně vrstvy. Zhodnoťte přežívání buněk a dlouhodobou retenci v daném tvaru. 4) Proveďte testy značení a vizualizace buněk a sběr mikroskopických dat. 5) Proveďte aplikaci buněk imunitního systému (leukocyty) do kultivačních jamek a analyzujte kontakt s vrstvou endoteliálních buněk. Analyzujte transmigraci leukocytů vrstvou endoteliálních buněk. 6) Proveďte diskusi získaných výsledků a zhodnoťte účinnost a využitelnost navrženého řešení.

DOPORUČENÁ LITERATURA:

[1] KNOPFOVÁ, L., et al. Možnosti studia transendoteliální migrace in vitro. Klinická onkologie. 2014, 27(1): S28-S33.

[2] CUCULLO, L., MARCHI, N., HOSSAIN, M. and JANIGRO, D. A dynamic in vitro BBB model for the study of immune cell trafficking into the central nervous systém. Journal of Cerebral Blood Flow. 2010, 31(2): 767-777.

Termín zadání: 8.2.2016 ***Termín odevzdání:*** 27.5.2016

Vedoucí práce: Larisa Baiazitova

Konzultant bakalářské práce: Josef Skopalík

prof. Ing. Ivo Provazník, Ph.D., *předseda oborové rady*

ABSTRAKT

Teoretická část této práce obsahuje biofyzikální popis endoteliální vrstvy a transmigrace buněk přes tuto vrstvu. Dále popisuje charakteristiky všech důležitých komponent pro vytvoření modelu cévy in vitro (endoteliální buňky, kmenové buňky, leukocyty, Calcein AM, PKH26).

Praktická část práce je věnována sestavení in-vitro modelů dvojrozměrných a trojrozměrných endoteliálních vrstev, jejich zobrazení pomoci mikroskopu a testování interakce leukocytů a kmenových buněk s těmito endoteliálními vrstvami.

V závěru je navrhnut nejlepší tvar a velikost mikrofluidních systémů simulujících kapiláry.

KLÍČOVÁ SLOVA

Fluorescence, konfokální mikroskopie, Calcein, PKH26, endotelové buňky, kmenové buňky, bílé krvinky, transendotelová migrace leukocytů.

ABSTRACT

Theoretical part of this study includes biophysical description of endothelial layer and cell transmigration through it. There is descripted characteristics of all important components used for creation of capillary model in vitro (endothelial cells, stem cells, white blood cells, Calcein AM, PKH26).

Practical part of the study devotes to fabrication of two-dimensional and three-dimensional in-vitro models of endothelial layers, processing of visualization by microscope and testing interaction between leukocytes, stem cells and endothelial layer.

In conclusion is proposed the best form and size of microfluidic models simulated capillary.

KEYWORDS

Fluorescence, confocal microscopy, Calcein, PKH26, endothelial cells, stem cells, white blood cells, transendothelial migration of leukocytes.

Bibliografická citace:

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Prohlášení

Prohlašuji, že svou bakalářskou práci na téma Simulace adheze a transmigrace imunitních buněk stěnou kapilár jsem vypracovala samostatně pod vedením vedoucího bakalářské práce a s použitím odborné literatury a dalších informačních zdrojů, které jsou všechny citovány v práci a uvedeny v seznamu literatury na konci práce.

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V Brně dne ....

podpis autora (autorky)

Poděkování

Ráda bych poděkovala páni Ing. Larise Baiazitově za vedení mé bakalářské práce, vstřícnost při konzultacích, odborný dohled, trpělivost a ochotu, kterou mi v průběhu zpracování práce věnovala. Zároveň děkuji panu Mgr. Josefu Skopalíkovi za poskytnutí buněk k měření a cenné rady, které mi pomohly tuto práci zkompletovat.

V Brně dne ....

podpis autora (autorky)

Obsah

1. Introduction

Modern researches in field of chemical compounds and microelectronic setup allow us to create and develop prototypes of biological systems, including in vitro models of blood vessels, simulator of interaction between blood cells and endothelial layer and simulation of trasmigration of the cell through the endothelial layer. Base on theoretical knowledge of these biological processes we can simulate their running and these simulations can help to discovery of the important aspect for medicine.

This bachelor thesis is prepped on theoretical and following practical research of 2D and 3D in-vitro experiments with endothelial cells and leukocytes; studying main principles of process leukocyte transendothelial migration; creating models, which simulate process of leukocyte transendothelial migration with and without circulation; visualization of transmigration process by cells labeling via Calcein AM and PKH26.

In the course of experiments created samples were scanned by laser confocal microscope LEICA TCS SP8. Study includes theoretical description principles of confocal microscopy and definition of suitable size, form and analyze of physical and chemical properties of sample's compounds.

In conclusion of that study is requested suitable at all aspects model, attested by experiment way. Achieved results are processed with proposed solution for statistical assay in Matlab software.

2. Fluorescence

Atoms are located either in the base energy state with a minimum value of electrons energy or in excited states. Excitation occurs when atoms receive energy and electrons start to reserve higher energy levels (Fig. 1). This excited state is not constant, the change in state as an excited state releases a quantum of energy leads to de-excitation. In this process atom exposes quantum of electromagnetic radiation, which is equal to a difference between energy levels of electron. This effect is known as luminescence, according to its duration, luminescence is divided to fluorescence and phosphorescence. Fluorescence can be observed during initiation phase, but it immediately disappears right after it, the time of afterglow is usually about 8-10 seconds. Phosphorescence has a longer afterglow time than fluorescence (typically milliseconds to seconds), and therefore, it continues up even after initiation phase. [1] [2]

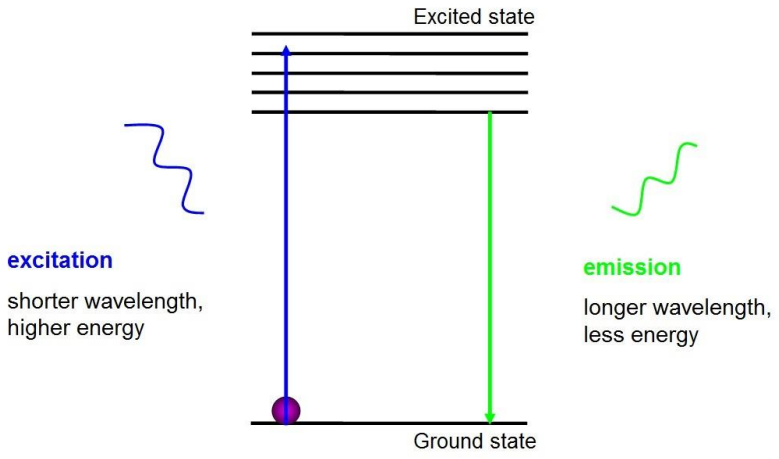


Figure 1. Excitation and emission. [Beatson Advanced Imaging Resource]

2.1 Principles of fluorescence

Fluorescence is appropriate for fluorescent dyes and fluorophores, which are molecules containing either aromatic hydrocarbons or heterocycles. Principle of fluorescence is illustrated by Jablonski diagram (Fig.2), which describes fluorescence as a three steps process consisting of initiation phase, excitation phase and emission.

The first step is the initiation phase, which begins with irradiation of the fluorophore by electromagnetic radiation of a definite wavelength from an external source, which usually is a light bulb or a laser. The emitted photon is absorbed by fluorophore and excitation occurs with transition of electrons from the base level to a higher energy level.

The second is excitation phase, which takes a very short time, generally from 1 to 10 nanoseconds. During this time fluorophore is subjected to different conformational changes and also mutually interacts with the molecular environment. The relaxed excitation state is achieved, when quantum of energy is reproduced to the environment in form of heat.

The third step is emission phase, when residual energy is dissipated in the form of fluorescent photons, which stimulate fluorophore to return to the basic state. However, all originally excited molecules will not reach basic state during fluorescence, because process can be break by internal conversion or fluorescence resonance energy transfer. [3], [[7] BEREZIN, Mikhail Y. a Samuel ACHILEFU. Fluorescence Lifetime Measurements and Biological Imaging. Chemical Reviews. 2010, 110(5), 2641-2684. DOI: 10.1021/cr900343z. ISSN 0009-2665. Dostupné také z: http://pubs.acs.org/doi/abs/10.1021/cr900343z

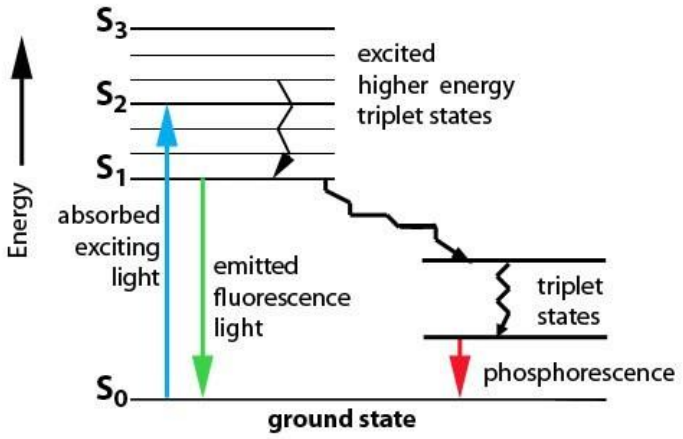


Figure 2. Jablonski diagram. [Beatson Advanced Imaging Resource]

2.2 Fluorophores

Fluorophores are molecules capable of fluorescence. The main condition of fluorophores is absorption of light of a certain wavelength with following ability of emission light with longer wavelength. Fluorochromes terminologically belong among fluorophores, they have same physical properties, and difference is that fluorochromes don't occur naturally, they can be only added synthetically. Fluorochromes are mostly used for marking biological material.

Fluorophores can be divided into two basic groups: internal and external. Internal fluorophores appear in nature and are capable of autofluorescence. Exterior fluorophores

are added to the samples, which have no capability for autofluorescence. Exterior fluorophores have wider and more frequent use in modern biology and biophysics than internal fluorophores. Depending on the way which they connect to the testing sample, exterior fluorophores are divided into fluorescent labels, probes and fluorescent indicators. Fluorescent labels are most commonly used for marking proteins. Attached by covalent bonding at proteins thiol, amine and sulfhydryl or histidine side chains. They are used in many applications in immunology, histochemistry, affinity chromatography, etc. Fluorescent probes attach to the sample by non-covalently bond and often change its fluorescent properties. There are thousands of probes, the right choice is crucial for experiments in fluorescence spectroscopy, because its features allow to obtain the necessary information. There are fluorescent indicators among fluorophores, which exhibit spectral sensitivity to certain substances. These fluorophores lead to a spectral shift depending on the presence of certain substances or exhibit an increase in fluorescence intensity without a spectral shift in the presence of the substance. In this study is used such fluorescent dye like Calcein AM and PKH26. [4]

2.3 Calcein AM

Calcein AM is a cell-permeant and non-fluorescent compound that is widely used for determining cell viability. Non-fluorescent Calcein AM in live cells is hydrolyzed by intracellular esterase into the strongly green fluorescent anion Calcein, and is retained in the cytoplasm of live cells. The excitation and emission wavelengths of Calcein are 495 nm and 516 nm, respectively (Fig. 3). [5]

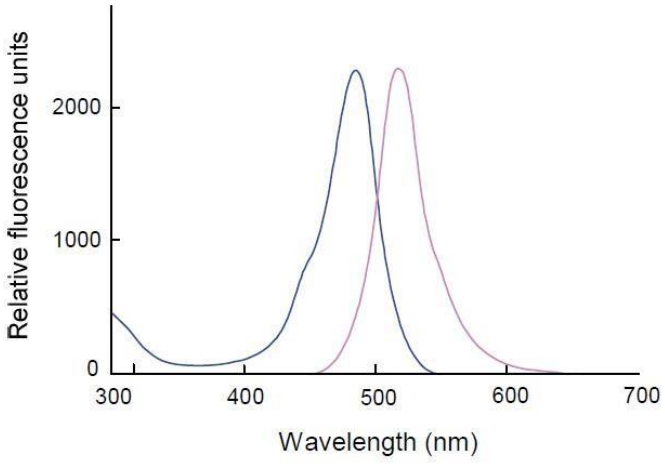


Figure 3. Fluorescence spectra of Calcein, AM. [ThermoFisher Scientific, Technical Bulletin, Calcein AM]

2.4 PKH 26

According to the technical bulletin provided by Sigma – Aldrich ® PKH26 is fluorescent cell linker kit, which uses proprietary membrane labeling technology and fluoresces in the yellow-orange region of the spectrum (Fig. 4). PKH26 fluorescence is independent of pH within physiologic ranges and fluorescence intensity per cell is typically unaffected by the pattern of dye localization. Components of PKH26 include PKH26 Dye, which is the cell linker dye, and Diluent C solution for maintain cell viability and staining efficiency during the labeling step.

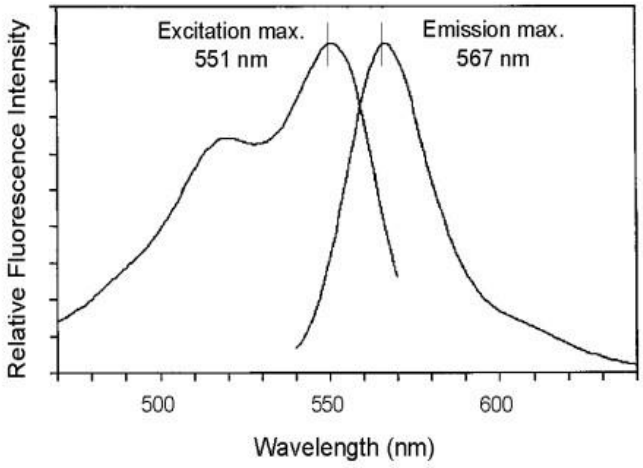


Figure 4. PKH26 Excitation and Emission Spectra. [Sigma-Aldrich, Technical Bulletin, PKH26]

One of the most common uses of PKH26 is proliferation analysis based on dye dilution. PKH26 has also proven useful for monitoring: uptake of exosomes viruses, platelets and other nanoparticles; apportionment of membrane in stem cell division; cell-cell membrane transfer; phagocytosis; antigen presentation; adhesion; signaling through gap junctions and neuronal migration in tissue slices. [6]

2.5 Fluorescence lifetime and autofluorescence

The fluorescence lifetime is defined as the average time that molecule spends in the excited state prior to returning to the ground state. Return to the ground state leads to emission of fluorescent photons. Generally, the fluorescence lifetime is about 10^{-9} s (Fig.5).

Fluorescence lifetime doesn't depend on the concentration of fluorophore absorption, the thickness of the sample and the intensity of excitation, but on the biological properties such as pH, temperature and structure of fluorophore, concentration of oxygen, changes of environment or a bond with different molecules.

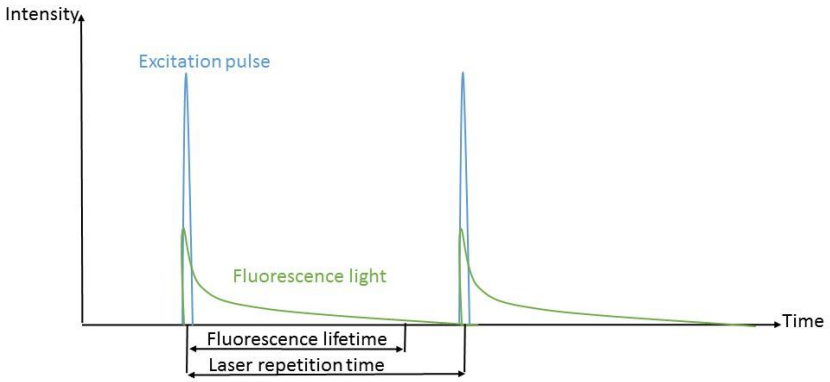


Figure 5. Illustration of the fluorescence lifetime principle: short-pulsed excitation light (blue) and the longer time duration fluorescence emission light (green) is shown as a function of time.

Some biological compounds have ability of intrinsic fluorescence, which is known as autofluorescence. Autofluorescence is self-induced fluorescence – emitting of light during absorption of radiation of some other wavelength. Primarily autofluorescence importance for the study of cells is in its potential for diagnostic applications and also as a research tool for understanding the basic mechanisms of molecular interactions and signaling processes in their natural conditions. The most important autofluorophores in cells and tissues are amino acids, the building blocks of proteins and enzymes, which regulate cellular metabolism. Imaging of autofluorescence is an attractive method because it does not require either marking of cells during in vitro experiment or adding of exogenous molecular agents in vivo. However, the complex nature of autofluorescence is associated with numerous problems including difficulty in interpreting of results. [7], [8]

2.6 Confocal microscopy

The confocal microscope has its name from the arrangement of the light path (Fig. 6). The basic principle is based on the fact that light coming through the focal point of the objective lens gives a sharp image. Light coming through another point than the focal is also shown, but image is not sharp. In the confocal microscope is used diaphragm point (pinhole) which filters blurred image. Pinhole must be conjugated with the focal point of the lens thereby is obtained confocal pinhole. In case if pinhole would be smaller useful light is lost, in case if pinhole would be larger light is coming out of focus. From the arrangement of a confocal microscope comes, that on the first step we get information only about one point, and therefore to obtain the entire image, it is necessary to scan point by point whole sample. According to the mechanism of rasterization there are two basic methods - scanning confocal microscopy and a confocal microscopy with spinning disk. [9]

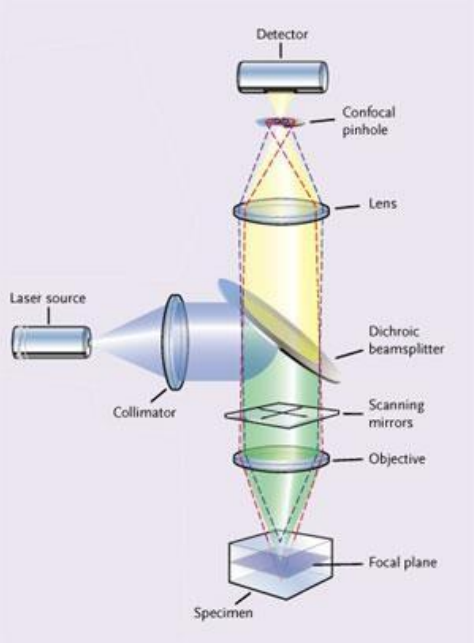


Figure 6. Scheme of confocal microscopy scanning. [TheSceintist, The Confocal Microscope]

2.7 Laser Confocal microscope LEICA TCS SP8

The Leica TCS SP8 is the most advanced confocal microscope. It is characterized by high speed, optimal efficiency and high contrast of obtained images with saving life abilities of scanned cells. For detection is used a very sensitive high speed scanning system that is able to receive up to 428 images per second, and also a large field of view enables to explore even the most demanding samples. The light source is a white pulsed laser, which allows set up to 200 different excitations. The advantage is the possibility set up to eight wavelengths simultaneously. Combining excitation using the white pulsed laser and detection using hybrid detectors is possible to combine the approximately 200 million variations of excitation and emission. Leica TCS SP8 is further equipped with acoustic-optical beam splitter, which is able quickly switch and debug individual wavelengths, without influence to emission of the important signal. Next advantage is the pulse mode white pulsed laser, whose clock frequency is exactly suited to the study of fluorescence lifetime, which is typically from 0,5 ns to 5,0 ns. The intervals between pulses can be changed, making the Leica TCS SP 8 useful tool for measuring fluorescence lifetime. [10]

3. Endothelial cells and blood vessels

Endothelium is the single-layer type of epithelium forming endocrine organ, diffusely distributed to the body's tissues, lines the cavities of the heart, the serous cavities, and the lumia of the blood and lymph vessels. Endothelial cells have the amazing ability to change its quantity and location according to the local requirements by

expression molecules capable to recognize leukocytes circulating the bloodstream, thus providing their adhesion, as well as the allocation in the vascular system. [11], [12]

3.1 Origin of endothelium

Endothelial cells arise from haemangioblasts, blast-like bipotential cells. Precursor cells come into being from the ventral floor of the dorsal aorta. Splanchnopleuric mesoderm transforms into mesenchymal cells, which differentiate into the haemangioblasts (Fig. 7). The haemangioblast then becomes an intermediate pre-endothelial cell, which can further differentiate into either a committed haematopoietic cell line, or an endothelial cell. [12]

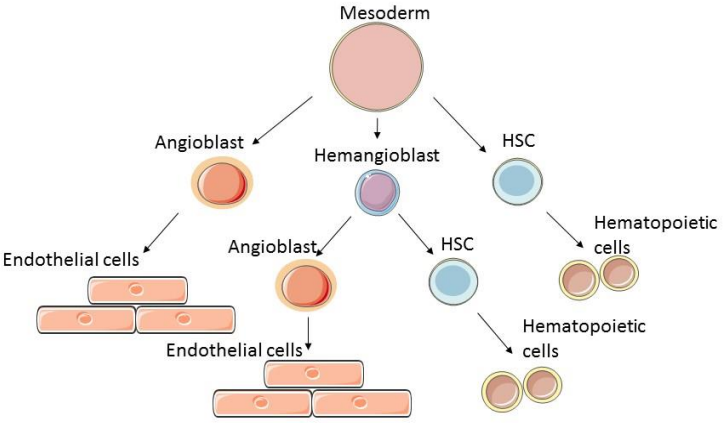


Figure 7. Scheme of origin of endothelial cells. [Servier medical art]

3.2 Classification and structure of blood vessels

Blood vessels are circuits through which blood is delivered to body tissues. The vessels make up two closed systems of tubes that begin and end at the heart. The pulmonary vessels system transports blood from the right ventricle to the lungs and back to the left atrium. The systemic vessels system, carries blood from the left ventricle to the tissues in all parts of the body and then returns the blood to the right atrium.

There are three types of blood vessels: arteries, capillaries and veins, which differ from other by function and structure (Fig. 8). The largest blood vessels are arteries and veins, which have a thick solid wall of connective tissue and smooth muscle. This wall is lined inside with extremely thin single-layer of endothelial cells, which is separated from the surrounding layers by basement membrane. The thickness of the connective tissue and muscle layers of the wall varies depending on the diameter of the vessel and the function, but the endothelial lining is always present (Fig. 9). The walls of the finest vascular tree

branches - the capillaries and sinusoids - consist solely of endothelial cells and the basement membrane.

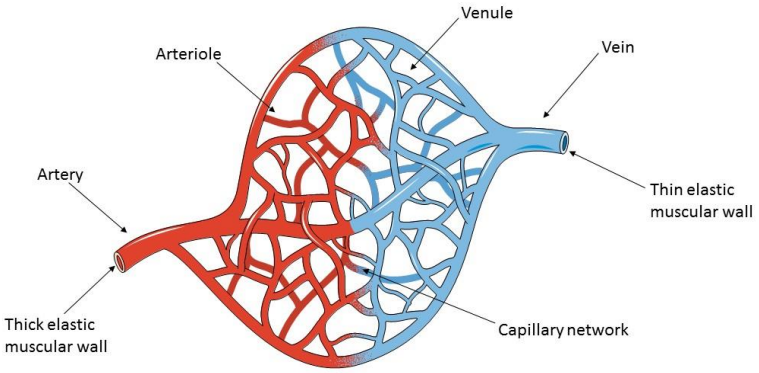


Figure 8. Blood vessels‘s classification. [Servier medical art]

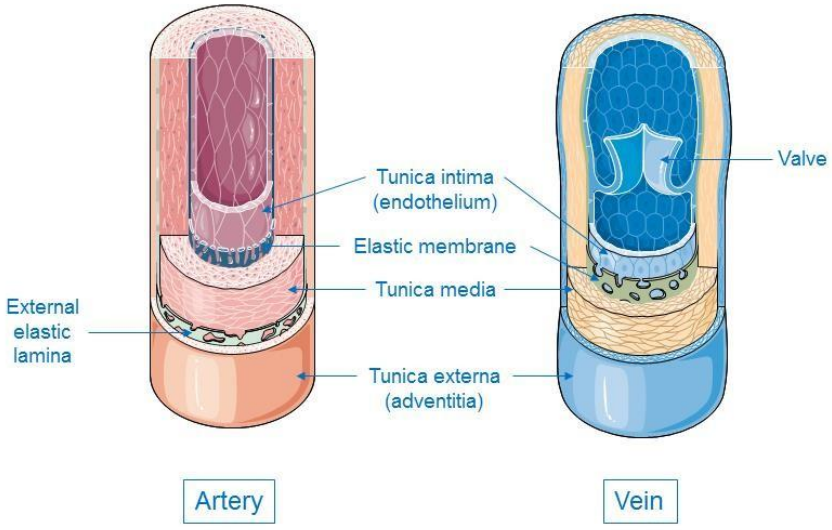


Figure 9. Blood vessels‘s structure. [Servier medical art]

Arteries carry blood away from the heart. Pulmonary arteries transport blood that has a low oxygen content from the right ventricle to the lungs. Systemic arteries transport oxygenated blood from the left ventricle to the body tissues. Blood is pumped from the ventricles into large elastic arteries that branch repeatedly into smaller and smaller arteries until the branching results in microscopic arteries called arterioles. The arterioles play a key role in regulating blood flow into the tissue capillaries. About 10 percent of the total blood volume is in the systemic arterial system at any given time.The wall of an artery consists of three layers. The innermost layer, the tunica intima (also called tunica interna), is simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibers. The middle layer, the tunica media, is primarily smooth muscle and is usually the thickest layer. It not only provides support for the vessel but also changes

vessel diameter to regulate blood flow and blood pressure. The outermost layer, which attaches the vessel to the surrounding tissue, is the tunica externa or tunica adventitia. This layer is connective tissue with varying amounts of elastic and collagenous fibers. The connective tissue in this layer is quite dense where it is adjacent to the tunic media, but it changes to loose connective tissue near the periphery of the vessel.

Capillaries, the smallest and most numerous of the blood vessels, form the connection between the vessels that carry blood away from the heart (arteries) and the vessels that return blood to the heart (veins). The primary function of capillaries is the exchange of materials between the blood and tissue cells. Capillary distribution varies with the metabolic activity of body tissues. Tissues such as skeletal muscle, liver, and kidney have extensive capillary networks because they are metabolically active and require an abundant supply of oxygen and nutrients. Other tissues, such as connective tissue, have a less abundant supply of capillaries. The epidermis of the skin and the lens and cornea of the eye completely lack a capillary network. About 5 percent of the total blood volume is in the systemic capillaries at any given time. Another 10 percent is in the lungs. Smooth muscle cells in the arterioles where they branch to form capillaries regulate blood flow from the arterioles into the capillaries.

Veins carry blood toward the heart. After blood passes through the capillaries, it enters the smallest veins, called venules. From the venules, it flows into progressively larger and larger veins until it reaches the heart. In the pulmonary circuit, the pulmonary veins transport blood from the lungs to the left atrium of the heart. This blood has a high oxygen content because it has just been oxygenated in the lungs. Systemic veins transport blood from the body tissue to the right atrium of the heart. This blood has a reduced oxygen content because the oxygen has been used for metabolic activities in the tissue cells. The walls of veins have the same three layers as the arteries. Although all the layers are present, there is less smooth muscle and connective tissue. This makes the walls of veins thinner than those of arteries, which is related to the fact that blood in the veins has less pressure than in the arteries. Because the walls of the veins are thinner and less rigid than arteries, veins can hold more blood. Almost 70 percent of the total blood volume is in the veins at any given time. Medium and large veins have venous valves, similar to the semilunar valves associated with the heart, which help keep the blood flowing toward the heart. Venous valves are especially important in the arms and legs, where they prevent the backflow of blood in response to the pull of gravity. [13]

3.3 Functions of endothelial cells

Endothelial cells have both metabolic and synthetic functions. In this work we will focus to endothelial cells of blood vessels and models of these systems. Endothelial cells in blood vessels have two main roles:

I. A role in controlling a blood-tissue interface and regulate thrombosis, thrombolysis, platelet adherence, vascular tone, vascular permeability, leukocyte trafficking and blood flow. The endothelium is indispensable for body homeostasis; an uncontrolled endothelial cell response is involved in many disease processes, including atherosclerosis, hypertension, pulmonary hypertension, sepsis and inflammatory syndromes. [14]

II. The second main function of the endothelium is to regulate solute and macromolecule transfer across the blood vessel wall. The endothelium forms are an important semi-permeable barrier to the free passage of molecules and cells from the blood to the underlying interstitium and cells. Endothelial cells are coupled by tight junctions, which act as a selective barrier to the passing out of molecules from the circulation. [14]

Structural heterogeneity of the endothelial cells implies forming the shape of endothelial structure with help of various amounts of structural component of the endocytic pathway, according to task in which endothelial cells are involved. [14]

Table 1. Physical parameters of blood vessels.

Type of blood vessels	Total cross-section area [cm^2]	Blood velocity [cm/s]	Blood pressure [Pa]
Aorta	4.5	30.00 – 40.00	14.0
Arteries	20.0	10.00	13.4
Arterioles	400.0	0.50 – 1.50	4.0 - 10.7
Capillaries	4 500.0	0.05	2.7 - 4.0
Vein	40.0	54.00	0.3 – 2.7
Vena cava	18.0	8.00	0.0 - 0.3

There are several types of transport through endothelial layer:

- movement from the intestinal lumen into the blood;
- transport by caveolaes, which are invaginations in the cell membrane;
- paracellular transport by intracellular tight junctions. [12], [14]

3.4 Summary

Endothelial cells play key role is in the control of blood fluidity, platelet aggregation and vascular tone. Endothelial cells are possessed of remarkable plasticity. One can make the assumption that each one of the trillion endothelial cells included in our body is phenotypically distinct since, like a chameleon, each one has to sense and adapt to the needs of the various neighboring cells and to many different microenvironments.

Endothelial cells have finally emerged as key immunoreactivity cells involved in host defense and inflammation. These cells both produce and react to a wide variety of mediators including cytokines, growth factors, adhesion molecules, vasoactive substances and chemokines, with effects on many different cells. Endothelial cells are also intimately involved in the manifestations of infection, atherogenesis, hypertension and cancer. [12], [14]

4. Stem cells

Stem cells are undifferentiated cells of a multicellular organism, which is capable of giving rise to indefinitely more cells of the same type, and from which certain other kinds of cell arise by differentiation. All stem cells possess two essential properties: self-renewal and potency to differentiate into specialzed cells (muscle, bone, neurons, etc.).

Self-renewal is the ability to maintain unchanged phenotype after numerous cycles of cell division. There are two mechanisms to ensure that a stem cell population is maintained: obligatory asymmetric replication, when a stem cell divides into one mother and one daughter cell, mother cell is identical to the original stem cell, and daughter cell is differentiated; and stochastic differentiation, when one stem cell divides into two differentiated daughter cells.

According to the differentiation potential, stem cells are divided into several types (hematopoietic stem cells, neuronal stem cells, embryonic stem cells, etc.). Mesenchymal stem cells (MSC) represent one of the most interesting type of stem cells, because these

cells are not only resident in some specific tissue, but they can activate, removed to blood circulation and make so called “homing” in damaged tissue and start the regeneration.

In our study we used multipotent mesenchymal stem cells. These cells are used for transplantation in clinical studies. They are commonly injected to the circulation and they are able to sense some specific chemoattractants and transmigrate over endothelial layer into the pathological tissue (infarcted muscle, intestine inflammation and others). [11]

4.1 Mesenchymal stem cells

Mesenchymal stem cells are stem cells with differentiation potential, found in connective tissue. Mesenchymal stem cells can differentiate in a large variety of human tissues including osteogenic, chondrogenic, adipogenic and neuronal lineages (Fig. 10). [11]

MSC are used in modern transplantation programs. One of the most important use is modern experimental application of MSC into the coronary vessels in heart of patient with infarct of myocardium. Their migration across the endothelial layer of blood vessel and next migration to ischemic muscle and their paracrine activity have significant impact to regeneration of ischemic region with high number of death muscle cells. Many experimental works have shown that landing of mesenchymal stem cells to wall of blood vessel near ischemic region and communication with endothelial cells and transmigration across endothelial wall is very critical step of these type of cell therapy. This transmigration process was discovered after histological analysis, but the kinetic is still unclear and description of detail mechanism need additional analysis under high resolution. [15]

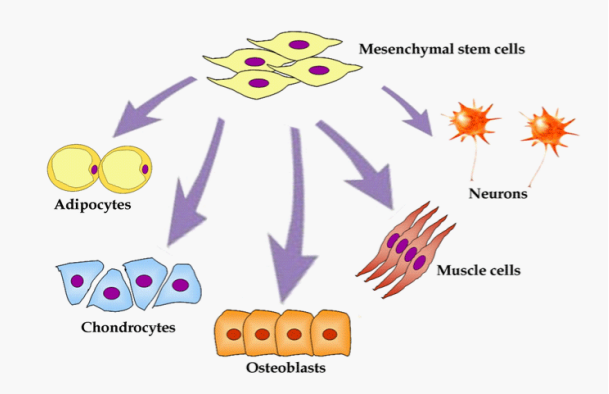


Figure 10. Mesenchymal stem cells. [Servier medical art]

5. White blood cells

White blood cells or leukocytes are an active part of the immune system of the body. Most white blood cells are made in the bone marrow known as a hematopoietic stem cell. Leukocytes are found in the blood and lymph tissue.

Categories of white blood cells can be divided into the five main types: neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Leukocytes are actively engaged in the destruction or neutralization of invading microorganisms and are quickly transported to the vicinity of infection or inflammation, so that they can move through the blood vessel wall to reach the site of injury. [11]

5.1 Chemotaxis of leukocytes

Process of the directional migration of leukocytes in response to chemical stimulus or external cues. Cell motility is vital in many physiological and pathological settings such as wound healing, cancer metastasis, and tissue remodeling, and it is a central phenomenon in reproduction, development, organ patterning, mobilization, and homing of stem and progenitor cells.

At the infection site, infected cells and the surrounding tissues release chemoattractants. These secreted chemoattractants form a concentration gradient and attract leukocytes, which move through the gradient towards the higher concentrations (Fig.11). The main function of chemokines is the induction of cell migration. Leukocytes will move toward the direction of increment of continuous chemokine concentration gradient. In other words, leukocytes migrate toward the source of chemokine. [16]

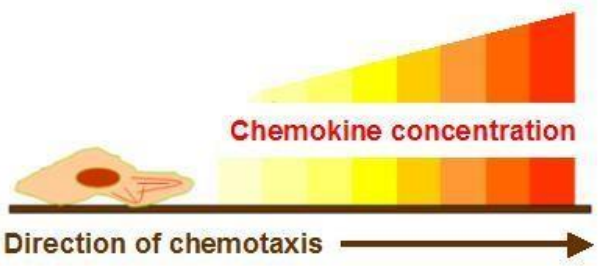


Figure 11. Chemotaxis gradient. [Wikipedia, Chemokine]

5.2 Transendothelial migration of leukocytes

The inflammatory response is the body's stereotyped reaction to tissue damage of any kind, evolved to heal wounds and fight infections. It involves rapidly delivering preformed soluble elements in the blood to the site of injury followed by a more prolonged

delivery of leukocytes. Leukocytes are recruited locally at the site of inflammation in a series of adhesive steps that allow them to attach to the vessel wall, locomote along the wall to the endothelial borders, traverse the endothelium and the subendothelial basement membrane, and migrate through the interstitial tissue. This multistep process of leukocyte transmigration can be dissected into a series of adhesive and signaling interactions between leukocytes and endothelial cells such as rolling, activation, adhesion and locomotion (Fig.12).

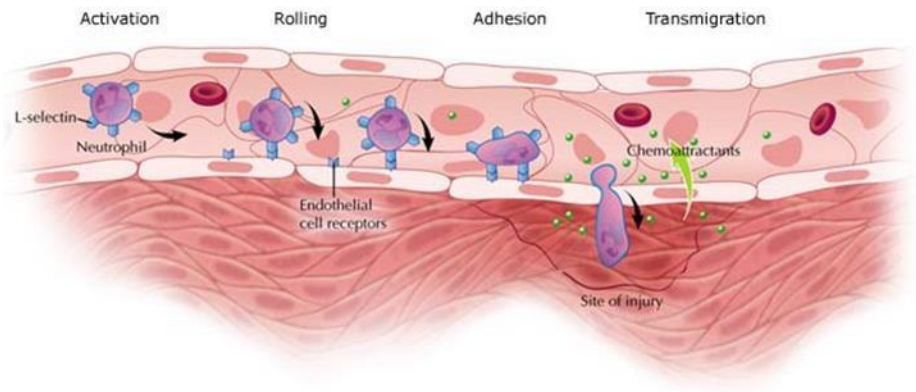


Figure 12. Cell transmigration. [StudyBlue, FloridaDaytona State Colleg, Diapedesis migration]

Rolling brings the leukocytes close enough for their chemokine receptors to be activated by chemokines attaches to the endothelial surface. Activated cell-surface receptors signaling cascade, activate leukocyte integrins for tighter adhesion to endothelium surface. Then attached leukocytes locomote over the endothelial surface to reach the junction. The preceding steps of leukocyte rolling, activation, adhesion, and locomotion are all reversible, and most leukocytes that initiate contact with the postcapillary venule at the site of inflammation reenter the circulation. [16], [17]

6. Methods for studying cell migration

The availability of suitable methods for studying cell migration properties is crucial for understanding molecular basis of events. In vitro methods for studying cellular migration include simple two-dimensional tests (scratch-wound assay and an assay based on the effect of hepatocyte growth factor), and methods using the effect of chemotaxis (Dunn chamber, video microscopy, using of carriers with chemoattractant). In vitro methods for studies of cell migration include more complex systems based on the principle of Boyden chambers (transwell migration, invasive test analysis via xCELLigence system, and confocal microscopy). [18]

6.1 Dunn chamber

Method allows microscopic observation of cell migration as a response to presence of chemoattractant. The Dunn chamber consists of two concentric wells separated by a 20 μm glass bridge. Cells are seeded on a glass slide and placed inverted on top of the concentric wells. A gradient is created from the inner well to the outer well and the responses of cells can be visualized in the bridge region (Fig. 13). Movement cross the bridge is scanned by microscope. This methodology is helpful tool for studying chemotaxis. [18]

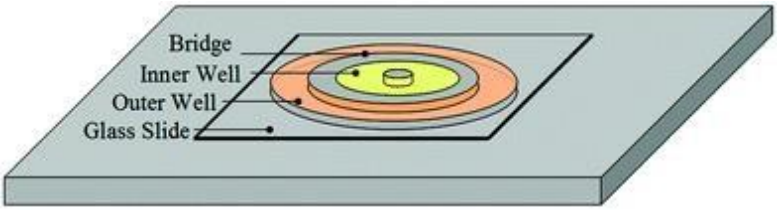


Figure 13. Dunn chamber. [Knopfová, Možnosti studia transendoteliální migrace in vitro. Klinická onkologie]

6.2 Boyden chamber

Method was originally developed for the study of leukocyte. Classical Boyden chamber (Fig. 14) consists of two compartments separated by a membrane. Cells with medium are situated at the top of the chamber and in the under part is a solution of cells with medium containing chemoattractant. The membrane between them is a physical barrier that can be overcome only by cells active movement. In this case we are talking about a three-dimensional (3D) environment, where collagen is used for covering of the membranes. Main advantage of this method is that in the 3D environment, the cells are polar, and therefore their behavior is closer to in vivo conditions. [18]

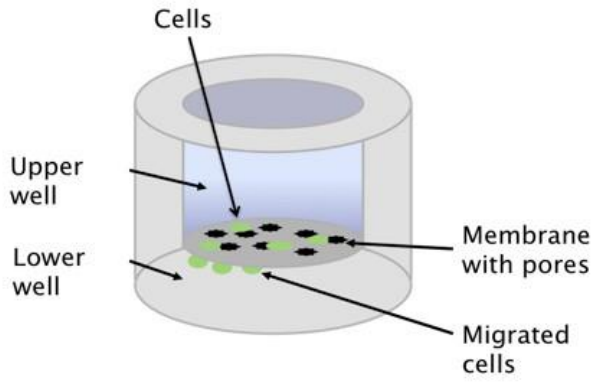


Figure 14. Boyden chamber. [Knopfová, Možnosti studia transendoteliální migrace in vitro. Klinická onkologie]

7. Basic principles of cell culture in vitro

Cell culture is the process by which individual cells are grown under controlled conditions in vitro.

Cells are grown in special culture media at a constant temperature, mammalian cells required special gaseous environment, which is supported by cell culture incubator, where concentration of carbon dioxide, water vapors and oxygen is regulated. For various cell cultures nutrient medium differ in composition, pH, glucose concentration, configuration of growth factors and others. One of the most important tasks during cultivation is to eliminate or minimize the application of contaminated ingredients.

Cells can be grown in suspension or in an adhesive state. For the cultivation of adherent cells is required a surface, such as tissue culture or plastic, coated with extracellular matrix components for improving adhesive properties and stimulating growth and differentiation.

Oversupply in the culture can occur due to a permanent cell division. This contributes to the following issues: accumulation of waste products in the medium, accumulation of dead cells in the culture.

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells. These are generally performed using tissue culture methods that rely on aseptic technique. Aseptic technique aims to avoid contamination with bacteria, yeast, or other cell lines. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. [19]

7.1 Chemical for better cell adhesion: Fibronectin and Poly-L-lysine

In our study was used two different natural polymers for better cell adhesion – Fibronectin and Poly-L-lysine.

Fibronectin is a multifunctional, extracellular matrix glycoprotein composed of two nearly identical disulfide bound polypeptides. Cellular Fibronectin is structurally and antigenically similar to cold insoluble globulin from plasma, therefore polyclonal antibodies to either form usually cross-react. Careful analysis of the Fibronectin molecule indicate that it contains several functionally and structurally distinct domains which may bind to cell surfaces, collagen, fibrinogen or fibrin, complement, glycosaminoglycan, proteoglycans and heparin. Numerous studies have shown that Fibronectin may enhance

cell adhesion and spreading and affect the routes of cell migration both in vivo and in culture. The extent of cell adhesion in vitro is related not only to the ability of the cells to interact with matrix-bound Fibronectin, when it is present, but also to the synthesis or lack of synthesis of Fibronectin by the cells, and to the lack of deposition of synthesized Fibronectin into an insoluble matrix surrounding the cells. Fibronectin is produced by a wide variety of epithelial and mesenchymal cells in vitro. Cellular Fibronectin is present in many tissues including spleen, lymph node, tonsil, blood vessel walls, liver, kidney, muscle, skin, brain and peripheral nerves. [20]

Poly-L-lysine is a polycation which binds to DNA, red cell membrane and any negatively charged protein. In cell cultures normal attachment, growth, and development of many cell types are dependent on attachment factors and extracellular matrix components. While some cells are able to synthesize these components, others require an exogenous source, particularly when grown in serum-free culture. Poly-L-Lysine is a charge enhancer; therefore, it can be used for coating many surfaces. [21]

8. Overview of modern studies with endothelial cells

In this part is presented modern experiments with endothelial cells in vitro and in vivo.

Human vascular endothelial cells: a model system for studying vascular inflammation in diabetes and atherosclerosis.

Endothelial dysfunction is recognized as initial step in the atherosclerotic process and is well advanced in diabetes, even before the manifestation of end-organ damage. Strategies capable of assessing changes in vascular endothelium at the preclinical stage hold potential to refine cardiovascular risk. In vitro cell culture is useful in understanding the interaction of endothelial cells with various mediators. Although circulating endothelial cells, endothelial microparticles, and progenitor cells opened the way for ex vivo studies. [22]

Development of an in vitro model for vascular injury with human endothelial cells.

The aim of study was to establish an in vitro screening assay for drug candidates using human endothelial cells as a model for vascular injury after intravenous application.

Different endpoints for viability and functionality of endothelial cells were investigated in human umbilical vein endothelial cells and in immortalized human endothelial cells. Cellular viability was determined by measuring ATP content and by the AlamarBlue assay. For comparison, the toxicity of the selected compounds was also tested in a murine fibroblast cell line. Selected endpoints for endothelial cell-specific function were vascular permeability, determined by measurement of the transendothelial resistance and the diffusion of tracer molecules, and the release of prostaglandin and thromboxane as indicators for prothrombotic or vasoconstrictor action. Five compounds (cyclosporine A, mitomycin C, menadione, amrinone and rolipram) were selected due to their known effects on the vasculature. The cytotoxicity of all compounds was similar in endothelial and 3T3 cells. ATP content and AlamarBlue metabolism did not differ significantly except for amrinone. A dose-dependent decrease of transendothelial resistance and an increase in FITC-dextran permeability could be measured in HUVEC cells for the tested compounds, but the sensitivity was not higher than that of the cytotoxicity assays. Increased prostaglandin or thromboxane release was detected for all compounds at cytotoxic concentrations and for rolipram also at non-toxic concentrations. [23]

In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis.

Discovery and comprehension of detailed molecular signaling pathways underlying endothelial vascular morphogenic events including endothelial lumen formation are key steps in understanding their roles during embryonic development, as well as during various disease states. Studies that used in vitro three-dimensional matrix endothelial cell morphogenic assay models, in conjunction with in vivo studies, have been essential to identifying molecules and explaining their related signaling pathways that regulate endothelial cell morphogenesis. [24]

Study of Platelet Behavior in Vivo after Endothelial Stimulation with Laser Irradiation Using Fluorescence Intravital Videomicroscopy and PEGylated Liposome Staining.

This study aimed to evaluate platelet behavior in vivo after endothelial stimulation with an argon laser, using video intravital microscopy in combination with a new and innovative platelet staining technique based on polyethyleneglyco liposomes. The study was performed on skin by using a dorsal skin-fold chamber implanted in golden hamsters. Platelets were stained by 5,6-CF-encapsulated PEGylated liposomes injected

intravenously. The skin microcirculation was observed with an intravital microscope (magnifications: ×25, ×40, and ×80) fitted with a xenon light source, an epifluorescence assembly, and an ultra-high sensitivity video camera for fluorescence imaging. Platelet activation without endothelial denudation or exposure of basal lamina and/or collagen was obtained with an argon laser emitting at 514.5 nm with the following parameters: 20 mW, 300 ms, 120 J/cm^2. The 80-µm laser beam was focused on a vessel and its position was controlled with the microscope (Fig.15). Thanks to the spatial resolution of the intravital microscopic imaging system, the platelets were seen rolling individually on the endothelium. After laser stimulation, platelets were activated and three phases were observed: recruitment, adhesion and detachment. The observation of these three phases was time dependent and the kinetics of the process were quantified. The recruitment reached a maximum after 90 ± 20 s. The adhesion phase lasted for 110 ± 25 s.

At last, detachment of all platelets was observed. This detachment started 200 ± 20 s after irradiation and was completed in less than 2 min. This study confirms that laser irradiation used with optimal parameters can induce platelet activation without thrombus formation. Platelets can adhere only transiently on stimulated endothelium. This phenomenon may therefore represent a defense mechanism, by which platelets would accumulate in the vicinity of an injury, making them available for immediate response. [25]

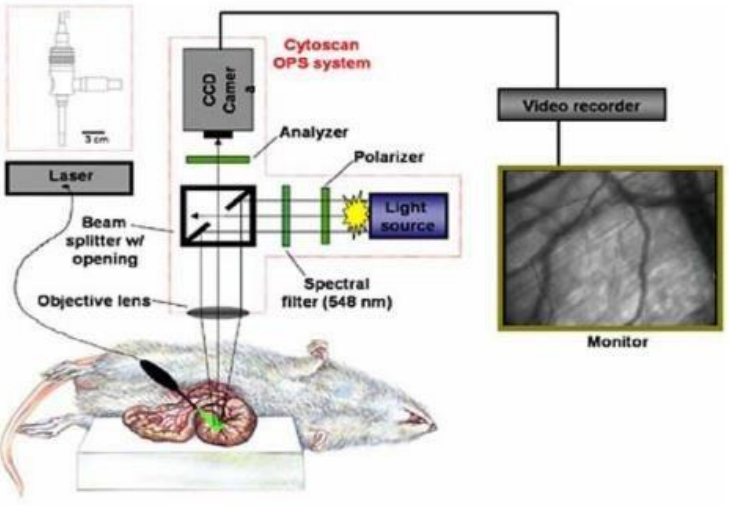


Figure 15. Diagram of the experimental setup. [Darkfield orthogonal polarized spectral imaging for studying endovascular laser-tissue interactions in vivo-a preliminary study]

8.1 Summary

In-vivo scanning and quantitative measurement of endothelial layer have been done more than fifty years, especially on small experimental animal. However, this in-vivo measurement must be done by very sophistic and high-cost microscope. These methods have many disadvantages including limited time period, problem with anesthesia of animal, impossibility of repeating of measurement on the same place of endothelium, and exigency of high number of animals. These disadvantages of in vivo experiments are main motivation for crating of endothelial layer and microcapillary structures in-vitro. Establishment of in-vitro simulator of endothelium – blood cell interaction, without needs of direct work with living animal. In last ten years several type of in-vitro model was crating on special smart cultivation chamber, many of them display perfect 3D geometry which is very similar with in-vivo situation, these modern smart tool are commonly called as "lab on chip" device.

In vitro system has still also some limitations and disadvantages. For example, the scaffold of endothelium is not so plastic as in vivo and not moving, also It's important to note that responses of cultured endothelial cells may not reflect responses seen in the same cells in vivo, and the immortalized endothelial cell lines used in many laboratory studies may, in particular, have altered expression patterns of key markers compared with cells studied in vivo. Also the circulating blood use in in vitro model device is almost not totally identical as real "living" blood, and setting of all chemical and physical parameters of this artificial blood flow is big challenge for the future biomedicine research.

9. Practical part of thesis

Practical part of the thesis devotes to the in vitro experiments with endothelial cells and leukocytes in 2D and 3D cultivation systems; processing of visualization via Calcein AM and PKH26; and analyzing of received results. In conclusion, microfluidic model with suitable parameters is proposed for scanning process of transendothelial migration by confocal microscope LEICA TSC SP8.

Practical part consists of series experiments. First experiment is aimed to creation of PDMS model (scaffold) for following application endothelial cells and testing adhesive properties and surviving and also compatibility of this final in vitro construct with confocal microscope (Chapter 9.1). Second experiment helps to define which kind of membrane can be used as base for creation of permeable scaffold for endothelial cells

grow and creating of planar cell layer. This model has higher similarity with real endothelial layer than PDMS scaffold, because space under endothelial cells is not rigid but clear for leukocyte migration. Determination of optimal initial endothelial cell seeding method, initial cell number for seeding and realization of control labeling of surviving cells by Calcein AM is an important part of second experiment (Chapter 9.4). In next step is approved which natural polymer creates attractive conditions for formation of endothelial cell layer distributed rather evenly over the surface of the model (Chapter 9.6). According to the achieved results in previous steps, in last point of practical part is devoted to construction of microfluidic 3D system (here not only endothelial cells but also real "blood" flow create good model of vessel).

9.1 Format of samples suitable for Laser Confocal microscope LEICA TCS SP8 X

To find out suitable format of samples, it's necessary to analyze properties of used materials and microscope's parameters.

Possible geometrical parameters of sample are coming from scanning range of microscope (limits of points which can be focused in x, y, z axis). Our confocal microscope Leica TCS SP8 X had limits of scanning volume, which is described in Fig. 16. During experiments was used objective 10X. For this type of objective and logical size 512px × 512px, scanning field has physical size 1160 µm × 1160 µm equally to x × y axes with z-step (destination between scanned slices) equal to 2.5 µm. Horizontal planes which can be focused are limited by limits of electronic z-moving of sample holder (limit is applied after fixed Z position of objective holder), this Z-limit is 1500µm. [28]

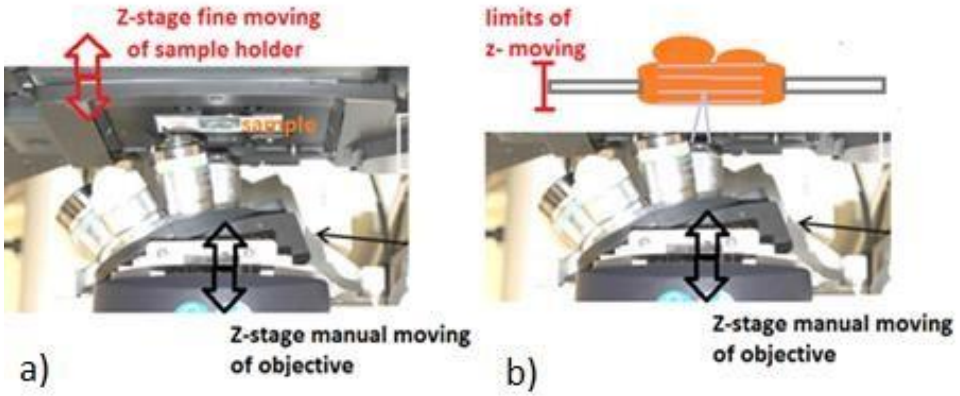


Figure 16. Moving of objectives and sample holder, limit of Z-moving: a) moving of sample holder before start of the scanning; b) objective holder is fixed, only sample holder is moving. [User Guide to the IBIF Leica TCS SP8 MP Confocal Microscope]

Next, there is a need to analyze properties of PDMS and molds, which were used during experiments (Tab.2). All mentioned parameters were indicated by data afforded by manufacturer. [26], [27]

Conclusion: suitable geometrical parameters of sample are based on scanning range of laser confocal microscope LEICA SP8, they are equal to 1160µm × 1160µm × 1500µm diapason on x × y × z axes. At the same time all used materials should guarantee satisfactory transparency.

Table 2. Material's properties.

Material	Description	Size (x; y; z) [mm]	Transparency [nm]	Other features
Petri Dish	Gama-sterile flat dish made of polystyrene	58 × 58 × 15	300 to 2500	Non-cytotoxic, non-pyrogenic, ideal for cell microbiology applications and confocal microscopy
PDMS	Polymeric organosilicon	58 × 58 × 15	down to 300	Colorless, transparent elastomer

9.2 Creating of PDMS channel

Goal of this experiment is to create model on basis of PDMS, which represent capillary. Experiment was divided to the few parts. Firstly, model of PDMS channel was constructed, and then endothelial cells were placed into the models. After 3 days of cultivation, cells were labeled via Calcein AM and scanned by laser confocal microscope. Adhesive properties of fabricated microchannels were analyzed in final part of experiment.

Method of fabrication PDMS channel: mixture of liquid PDMS and crosslinking agent is poured into the mold with tube and heated at high temperature. In first step of this

experiment was used half-tube. Once the PDMS is hardened, tube can be taken off the mold. We obtain a replica of the micro-channel on the PDMS block (Fig.17). [29]

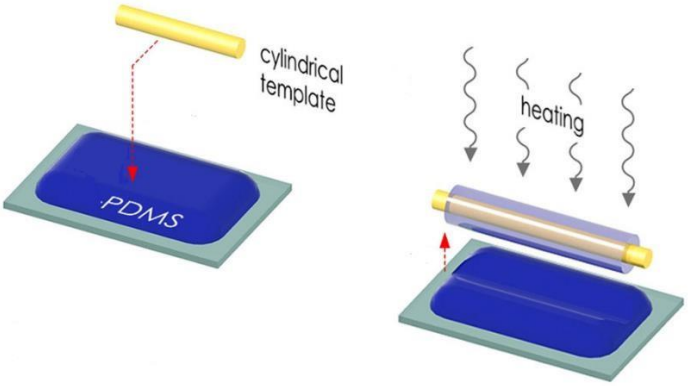


Figure 17. PDMS channel manufacturing. [Robot Globe, Micro-Robotic Tentacles]

Next step of experiment was dedicated to fabrication microchannel according to method described in previous part. For this experiment was used tube with smaller parameters for better representation of natural capillary system. Parameters of both tubes are shown in the Tab.3.

Table 3. Parameters of used tubes.

Sample	Length [mm]	Diameter [mm]
Tube 1	6.0	6.0
Tube 2	1.5	0.4

On the next part of experiment endothelial cells were placed into fabricated models. The circular PDMS microfluidic channel was sterilized with 70 % ethanol and exposed to UV overnight. Before addition, cells were trypsinized with 0.25 % trypsin for 10 minutes, concentrated by centrifugation, and then resuspended with the complete medium. Samples with endothelial cells were cultured and grown in a humidified 5 % CO_2 incubator at 37˚C. Cells were labeled via Calcein AM before scanning.

9.3 Procedure of labeling cells via Calcein AM

Reagent preparation: 1x Calcein AM DW Buffer - Dilute the 10x Calcein AM DW Buffer to 1x before use. For each 96-well microplate is used 5 mL of 10x Calcein AM

DW Buffer and 45 mL of deionized sterile water. Resultant concentration of Calcein AM is 10 µl.

Application of reagent: discard the media of cultured cell sample supplement and add 100 µL of 1x Calcein AM DW Buffer. Incubate for 30 minutes at 37° C under CO_2 (or normal culture conditions). 6. Record fluorescence using a 490 nm excitation filter and a 520 nm emission filter. The fluorescence intensity is proportional to the number of viable cells (Fig. 18). [30]

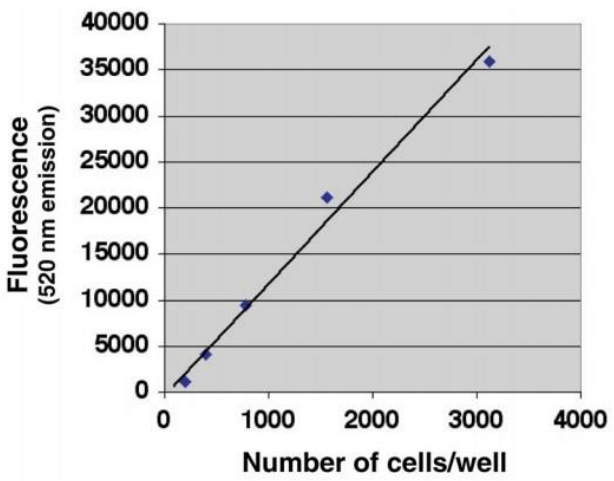


Figure 18. Proportional of fluorescence intensity to the number of cells. [ThermoFisher Scientific, Technical Bulletin, Calcein AM]

In final part of experiment both models filled up with cells were placed into laser confocal microscope LEICA TCS SP8 (Fig.19).

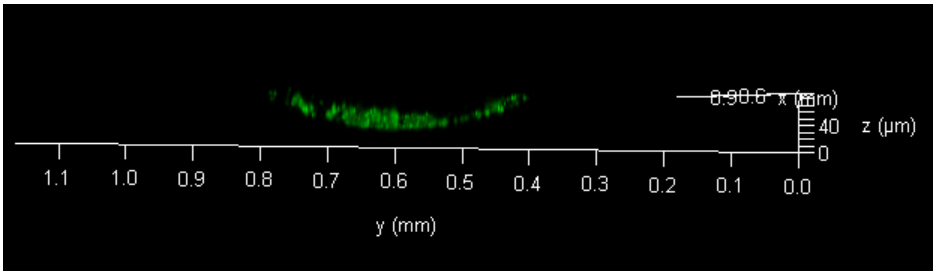


Figure 19. Endothelial cell layer on PDMS tube: endothelial cells (green dots), PDMS matrix (transparent). Scanned by confocal mode. 3D reconstruction from scanning procedure with z-step 2,5µm.

Conclusion: summing up the results, we could say, that endothelial cell layer does not cover surface of tube evenly enough, it generally situated at the bottom part of tube. That means, demonstrated model doesn't simulate real capillary system exactly. Moreover, growth of endothelial layer onto smaller tube does not turned out well.

However, first model show capability of adhesive properties that allow us PDMS as basis in next experiments.

9.4 Basic test of endothelial cells growth

Main aim of this experiment is to find out optimal parameters for creating model simulated capillary wall.

Description of experiment: At the first step of experiment, we search for the most appropriate material for cell adhesion and their long-time viability on its surface. We have tested several materials - membranes with different parameters. First tested material is product of BD Falcon nylon cell strainer with 100,0 µm pore size, the second - is nylon cell strainer product of BD Falcon with 40,0 µm pore size, and the last one is polycarbonate membrane the product of Corning Incorporated with 5,0 µm pore size (Fig.20).

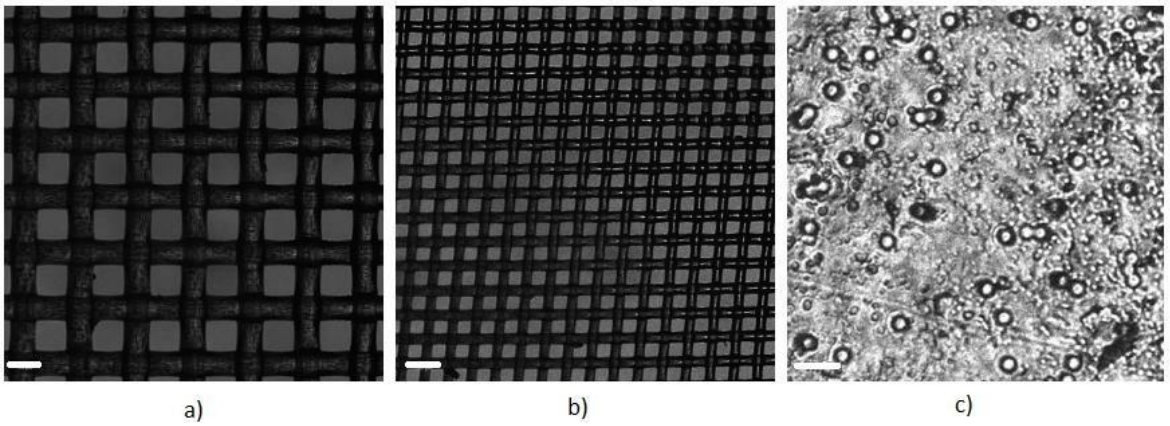


Figure 20. Structures of used membranes scanned by microscope: a) Nylon Cell Strainer 100,0 µm pore size. Scale bar: 100 µm; b) Nylon Cell Strainer 40,0 µm pore size. Scale bar: 100 µm; c) Polycarbonate Membrane with 5,0 µm pore size. Scale bar: 15 µm.

The same amount of mesenchymal cells was applied on enlisted bases, and then, cells were cultured and grown in a humidified 5 % CO_2 incubator at 37˚C. After 3 days of cultivation, cells were labeled via Calcein AM, and then samples with membranes were scanned by laser confocal microscope LEICA TCS SP8.

Conclusion: achieved results set, that among testing membranes adhesion was detected only on the surface of the last sample - polycarbonate membrane the product of Corning Incorporated with 5,0µm pore size. That result was expected, as description of product include manufacturer's notice about successful using that polycarbonate membrane for cells transmigration. [31]

According to the fact, that optimal range of cell number need to be optimized to ensure the best visualization of results, for polycarbonate membrane were prepared three samples included different initial quantity of cells on it. First sample contained 2,000 cells, the second 10,000 and the third 20,000. Results of search optimal cell number is represented in Tab.4. and Fig. 21. a, b, c.

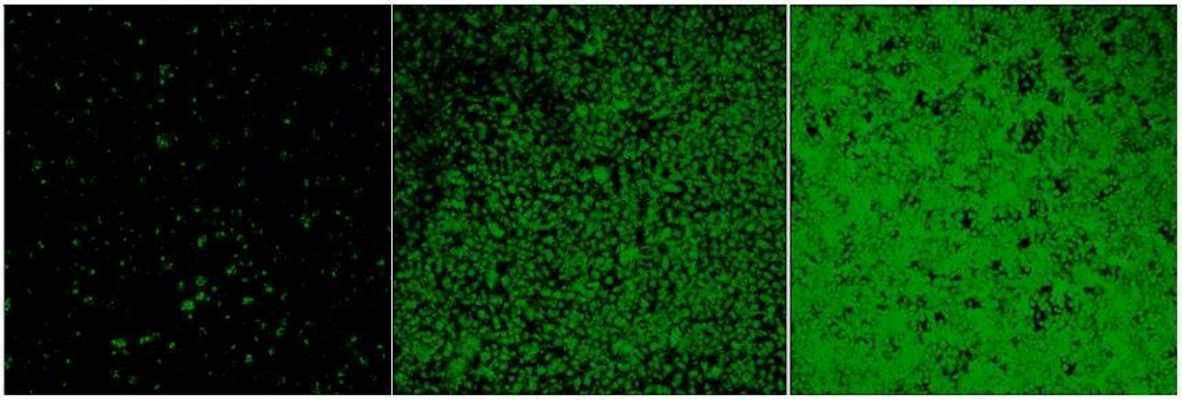


Figure 21. Samples with different initial cell's number a) 2,000; b) 10,000; c) 20,000. Images's definition is 1160µm x 1160µm.

Table 4. Cell's quantity of samples.

Sample	Initial quantity of cells	Total quantity of cells in the sample
a)	2000	10088
b)	10000	51546
c)	20000	98938

Conclusion: experiment showed that among testing quantities, the most optimal result demonstrate sample 2 with the initial quantity of added cells 10,000. The rest of samples demonstrate adhesion less clearly. On sample 1 cells aren't arranged close enough, and on sample 3 cells are too close to each other, forming a multi-level layer, both this samples complicate analysis and may falsify adhesive properties of model. Also, readily was observed, Calcein AM labels living cells (more brightly) and lifeless cells (dimly). Living cells have streamline oblong form, whereas lifeless cells have more angular form. That observation would be helpful in following experiments and calculations.

9.5 Endothelial cells calculation on models – developing of automatic script for image processing

1.Method

In the initial stage, software Fiji ImageJ was used for counting of cell quantity. Calculation was produced by two different methods. First method - manually, using the mouse and software's plugin cell counter, second method is automatic counting by optimally configured settings. In order to reduce the possibility of mistake, the productive value of cells quantity was taken as the arithmetic mean between resultant quantity values of both methods.

2.Method

In further stages, we use algorithm engineered in Matlab software. For every original image, obtained by laser confocal microscope, were scanned three equidistant field of area 1160 μm × 1160μm. For every field were made N slices with distance of 2,5 μm from each other.

Concept of algorithm: primary is made 3D image of every field by summing up all slices belonging to field. Obtained 3D images are converted to binary images. Trash hold value fixes in which intensity of grey color is recognize as white or black pixel. Trash hold values differ depending on cell type (endothelial or leukocyte), because of difference in coloring rate. Trash hold value (level) is installed manually by user. Next, binary images are processed by using morphological operators, such as bwmorth 'imdilate', 'imfill', 'imopen' and others. That step helps to correct cell's form and to clear off the noise (Fig. 22,23). User have to set structuring element (SE) according to a priori knowledge of cell appearance (endothelial cells have oblong form, leukocyte cells have round form, cell form is also depended on cell condition). Difference between endothelial and leukocyte cells form is represented on Fig. 24 a) and Fig.25 a)

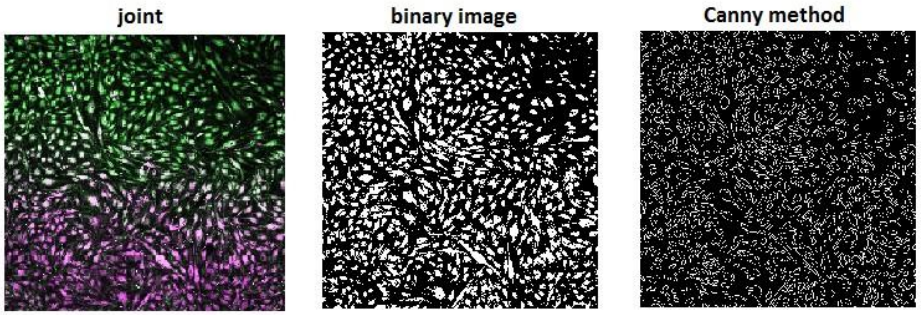


Figure 22. Steps of algorithm.

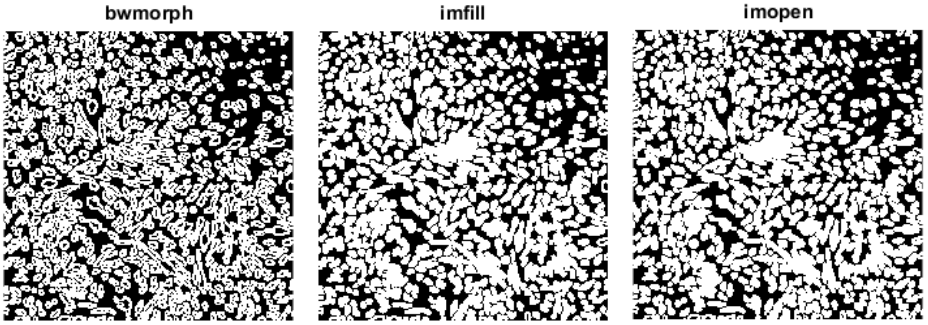


Figure 23. Steps of algorithm: morphological operations.

Algorithm calculate concentration as percent ratio of 1:0, where 1 represents white pixel, 0 represents black pixel (Fig. 24 b, Fig 25 b). At the first step we calculate concentration for every field, and then we define resultant concentration as mean value of concentrations of all scanned fields.

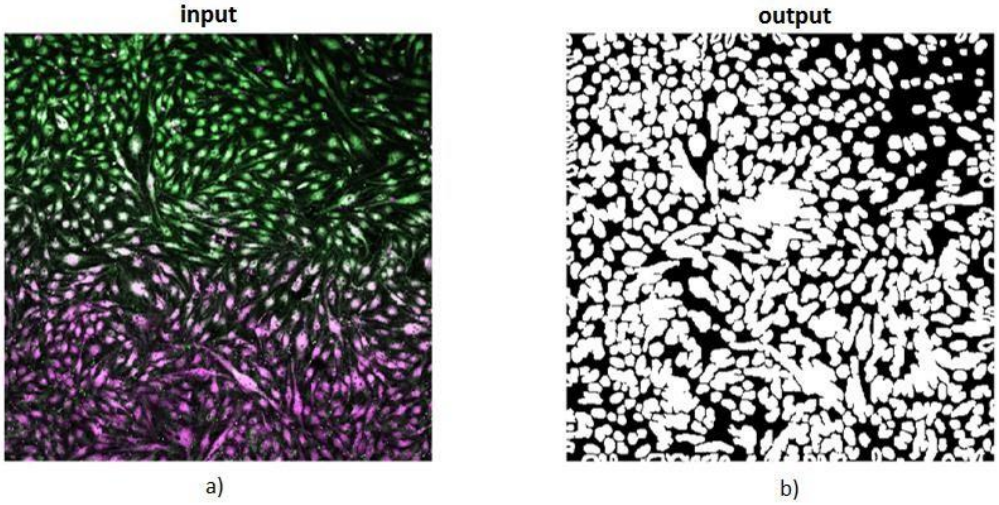


Figure 24. Endothelial cell layer: a) joint of N slices; b) processed binary output image.

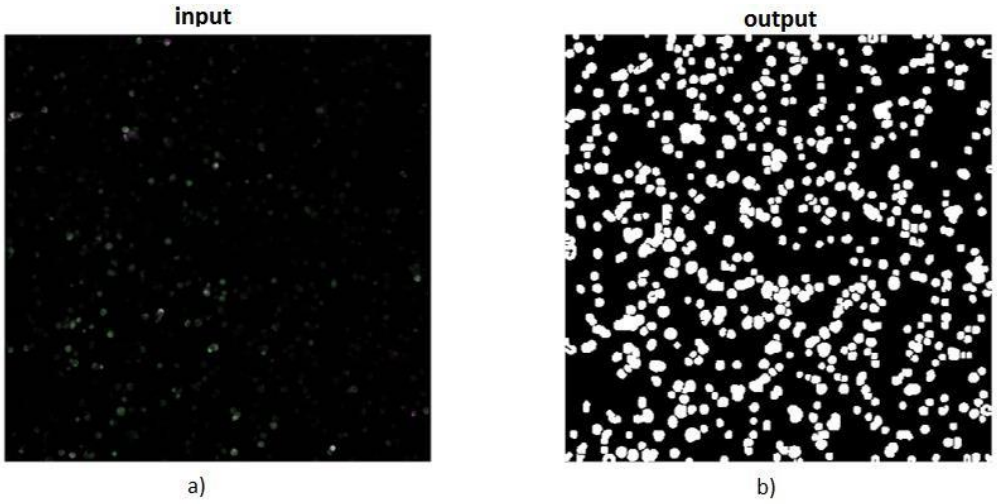


Figure 25. Leukocytes: a) input 3D rekonstruction image ; b) output binary image.

Conclusion: to compare both (1.method and 2.method) of cell calculation, we can resume, that Matlab algorithm is more appropriate for use in our case. Main advantage of that algorithm is possibility to change parameters of operators such as trash hold value of binary image (helps to find less coloring cells) and structure element (helps emphasize cell form) according to a priori knowledges of cells appearance in different states.

9.6 Search for suitable nature polymer

Goal of experiment is to define nature polymer, which creates attractive conditions for endothelial cells adhesion on the surface of PDMS model.

Method of experiment: for this experiment were created three models of PDMS channels:

- The first PDMS channel don't have any polymer, after sterilization with 70 % ethanol and disinfection by UV overnight, tube was filled up by 200 µl of DMEM medium contains 10,000 of endothelial cells.

- Surface of the second sample was coated with 0.1 mg/mL fibronectin by incubation at 37˚C for 1 hour, and then was tube was filled up by 200 µl of DMEM medium contains 10,000 of endothelial cells.

- Surface of the last channel was was coated with 0.1 mg/mL fibronectin by poly-L-lysin at 37˚C for 1 hour, and then was filled up with the same solution. After incubation period, endothelial cells were labeled by Calcein AM for visualization, and then samples were scanned via laser confocal microscope LEICA TCS SP8. Results of experiment are presented in Tab. 5. and Fig. 26.

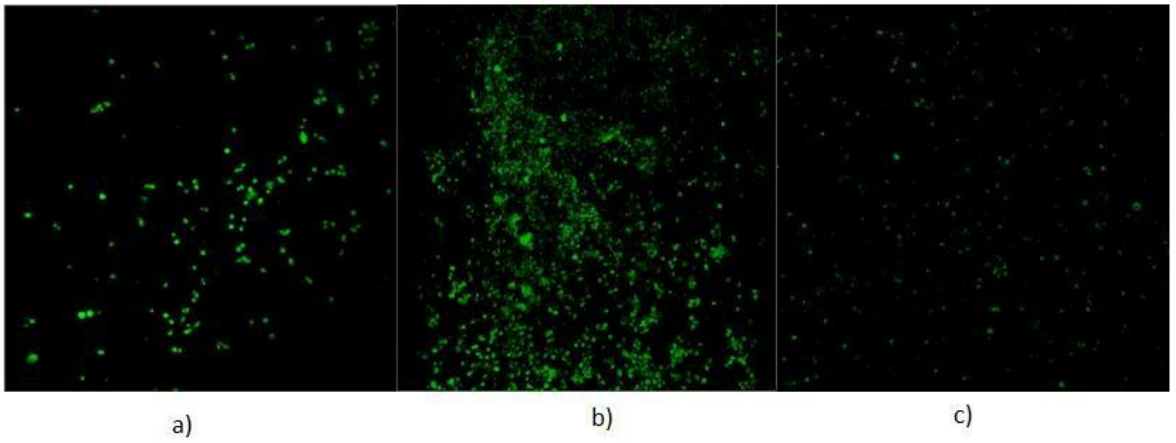


Figure 26. PDMS channels with different polymers: a) none; b) Fibronectin; c) Poly-L-lysine.

Table 5. Results of testing natural polymers.

Channel	Content	Number of adhezive cells per 1 mm^2 after period of cultivation
a)	PDMS channel without nature polymer	106
b)	PDMS channel covered by fibronectin	1005
c)	PDMS channel covered by poly-L-lysine	316

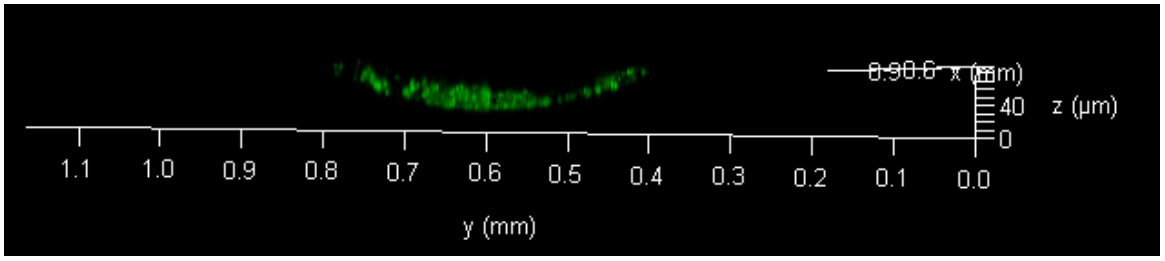


Figure 27. 3D vizualisation of endothelial layer on uncoated PDMS. Same parameters as at Fig.19.

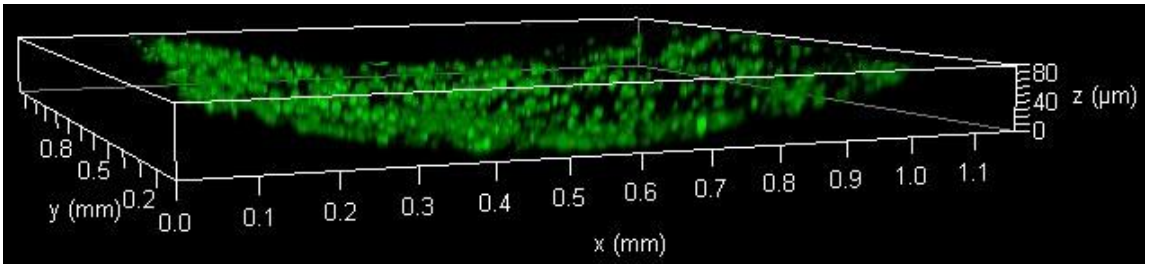


Figure 28. 3D vizualisation of endothelial layer on surface PDMS with Fibronectin (side view). Same parameters as at Fig.19

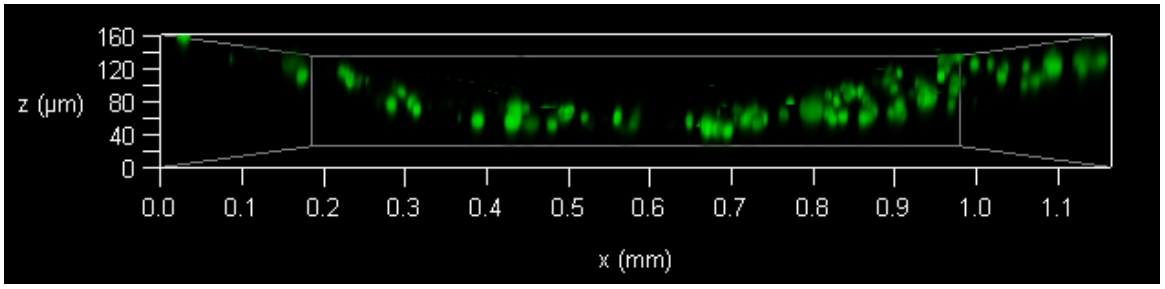


Figure 29. 3D vizualisation of endothelial layer on surface PDMS with Poly-L-lysine Same parameters as at Fig.19.

Conclusion: The experimental results show, that Fibronectin is the most suitable natural polymer, which creates attractive conditions for cell adhesion on the surface of PDMS channel, because endothelial cells cover the surface of the channel more tightly, than in case with Poly-L-lysine. We did not try to apply fibronectin to polycarbonate membrane, because porous structure of membrane provoke adhesion by itself as distinct to smooth structure of PDMS.

9.6 Creation of microfluidic 3D system with endothelial layer on membrane

Previous experiments show construct, where the endothelial cells are on solid matrix (PDMS). In this experiment we want create endothelial layer on porous membrane, which better mimics the real blood vessel. Goal of experiment: to create microfluidic 3D system according to the results derived from previous experiments and theoretical knowledges received from science studies. In this experiment, we use three different methods of fabrication: polycarbonate membrane with microfluidic nozzle, polycarbonate membrane integrated into the silicon tube, polycarbonate membrane integrated into the PDMS construct.

MODEL 1. Method: for manufacturing of 3D model was used polycarbonate membrane. In opposite walls of membrane were made two holes. To this holes were attached tubes by using of silicone (Fig. 30).

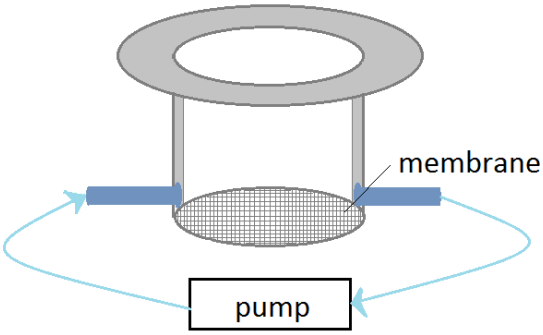


Figure 30. Schematic image of model 1.

Sample was sterilized with 70 % ethanol and exposed to UV overnight. Endothelial cells were trypsinized with 0.25 % trypsin for 10 minutes, concentrated by centrifugation, resuspended with the complete medium and added to the surface of membrane. Sample was cultured and grown in a humidified 5 % CO_2 incubator at 37˚C. After 3 days of cultivation, cells were labeled via Calcein AM, then model was scanned by laser confocal microscope LEICA TCS SP8.

MODEL 2. Method: model consist of tube and flat polycarbonate membrane tightly attached to the side (Fig. 31).

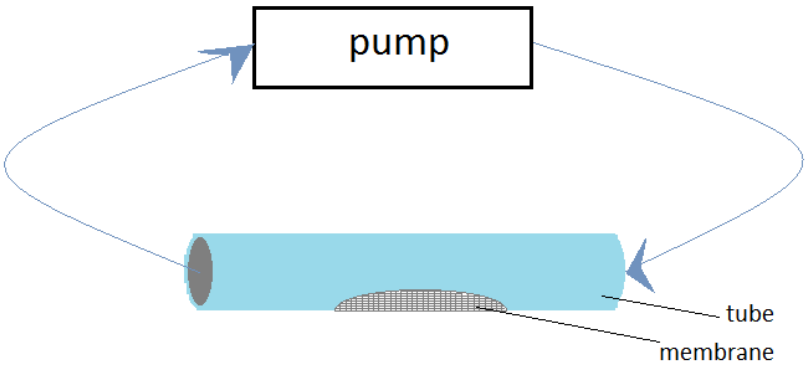


Figure 31. Schematic image of model 2.

Model was sterilized with 70 % ethanol and exposed to UV overnight. Endothelial cells were trypsinized with 0.25 % trypsin for 5 minutes, concentrated by centrifugation, resuspended with the complete medium and added to the surface of membrane. Sample was cultured and grown in a humidified 5 % CO_2 incubator at 37˚C. After 3 days of cultivation, cells were labeled via Calcein AM, then model was scanned by laser confocal microscope LEICA TCS SP8.

MODEL 3. Method: positive photoresist was spin-coated twice on the Si wafer to form a double layer of resist. Then, the double layered positive photoresist on the Si wafer was exposed to UV-light, post-baked, and developed. After that, positive photoresist was heated at 105 ˚C for 5 min to converting the rectangular shape to a circular shape. A PDMS prepolymer and PDMS curing agent, mixed at a volume ratio of 10:1, were poured into the convex half-circular photoresist on a Si wafer, and cured in an oven. The cured PDMS was then carefully peeled off from the Si wafer, and the resultant PDMS microchannel shape was a concave circle. The circular PDMS microfluidic channel was sterilized with 70 % ethanol and exposed to UV overnight. The surface of the circular microchannel was coated with 0.1 mg/mL fibronectin by incubation at 37˚C for 1 hr. After washing with Dulbecco's phosphate-buffered saline, the harvested HUVECs were introduced into the microchannel by using a micro-syringe pump with a flow rate of 14 nL/sec. After 10 min incubation in a humidified 5% CO2 incubator at 37˚C for cell attachment, the circular PDMS microchannel was turned upside down, and same amounts of HUVECs were introduced again. After another incubation period of 2 hours for complete and stable cell adhesion inside the entire circular microchannel, the chip was immersed in a petri dish containing a static media solution, which prevented any possibility of cell detachment or bubble formation during the cell culture (Fig. 32). [32]

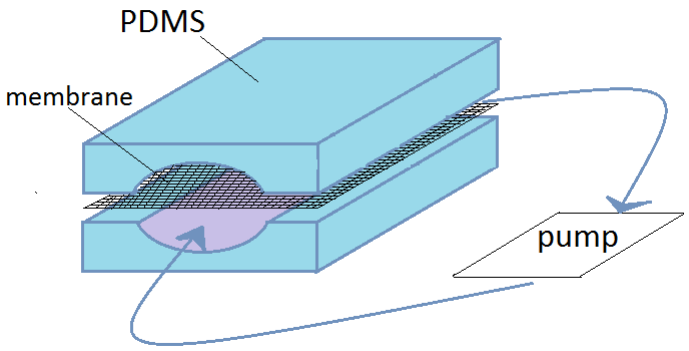


Figure 32. Schematic image of PDMS microfluidic channel.

Next stage of experiment consists in analyze of concentration of endothelial cell layer on the membranes' (after labeling via Calcein AM cells were placed into confocal microscope for scanning).

Model 1 presents relatively good result, the endothelial cells are viable (Calcein positive checked by microscope), cell layer is confluent. Model 2 (Fig. 31) have geometry with very good similarity to real capillary, unfortunately the surviving of cells is still not good. Probably there is the main problem with ideal disinfection of construct and

evaporation trough not ideal jointing. Model 3 is most difficult, the final test and scanning on microscope have not been finalized yet.

Based on achieved results, model 1 is more suitable for the next step. At the next point we have applied leukocyte cells labeled via PKH26 to the membrane of model 1 (Fig.33).

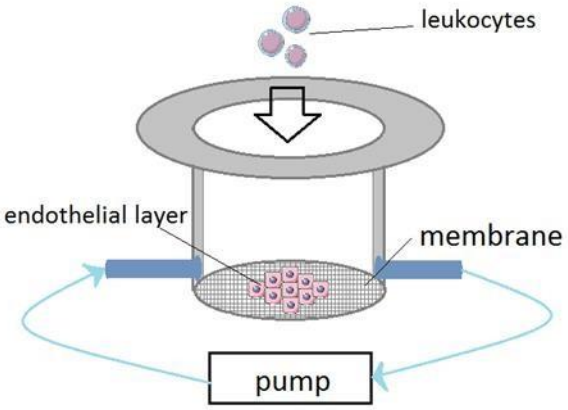


Figure 33. Schematic description of experiment.

9.7 Procedure of labeling cells via PKH26

Place cell suspension in a conical bottom polypropylene tube and wash once using medium without serum. (Serum proteins and lipids also bind the dye, reducing the effective concentration available for membrane labeling). Centrifuge the cells for 5 minutes into a loose pellet. After centrifuging cells, carefully aspirate the supernatant, being careful not to remove any cells but leaving no more than 25 mL of supernatant.

Prepare a 2x Cell Suspension by adding 1 mL of Diluent C to the cell pellet and resuspend with gentle pipetting to insure complete dispersion, then prepare a 2x Dye Solution by adding 4 mL of the PKH26 ethanolic dye solution to 1 mL of Diluent C in a polypropylene centrifuge tube and mix well to disperse. After that rapidly add the 1 mL of 2X Cell Suspension to 1 mL of 2X Dye Solution and immediately mix the sample by pipetting. [6]

9.8 Calculation of membrane's permeability. Simulation of blood flow with free circulation cells

At this chapter we apply fluidic medium with leukocyte and MSC to analyze the interaction of these circulating cells with endothelial layer (cell adhesion, cell migration).

Before application of leucocytes and MSC, it was necessary to stain these cells by the fluorescent labels, because nonfluorescent cells could not be detected in confocal microscope. PKH26 was selected as the most proper label (datasheet specification describe PKH26 fluorescent spectrum with maximum 567 nm, which is well separated from 520 nm fluorescence of Calcein). Method for staining cells is described in Chapter 9.7.

After staining, leukocytes were applied to the model 1 without circulation for setting transmittance of membrane (Fig.33). Initial state of leukocyte transendothelial migration is shown at Figure 34. Visible adhesive red fluorescence leukocyte situated on the horizontal of the endothelial layer (scanning settings of microscope: z-step 2,5µm, logical size 512px × 512px, physical size of scanning field is1160 µm × 1160 µm).

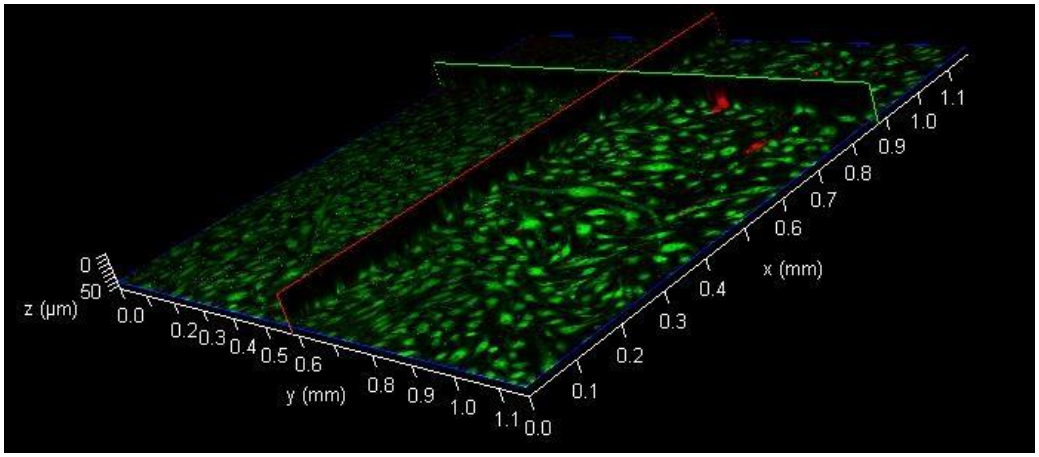


Figure 34. Leukocyte cells (red) on endothelial layer (green) (view from above).

After scanning of initial state, model was cultivated during 2 days, and then scanned again at its final state with the same settings of scanning (Fig.35).

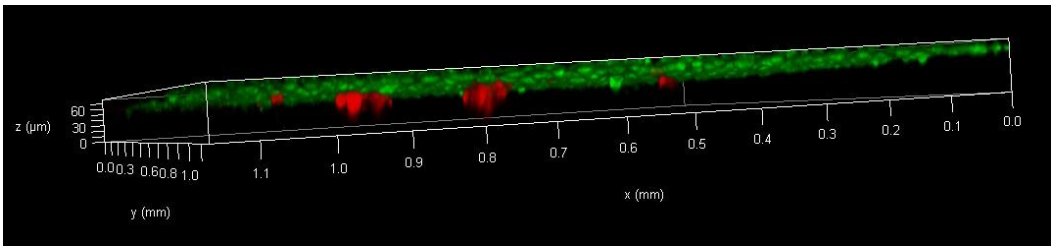


Figure 35. Leukocyte migration through endothelial layer (bottom view).

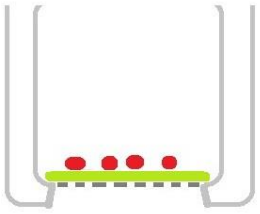
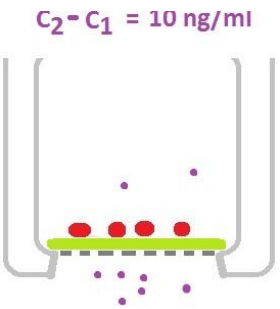
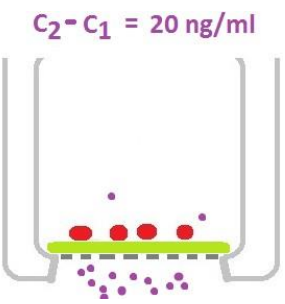
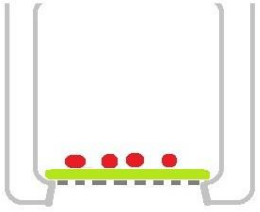
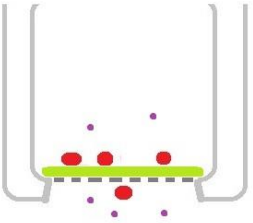
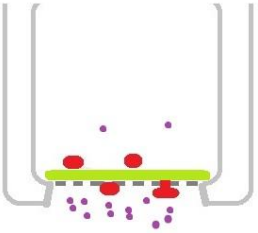
Transmittance of polycarbonate membrane covered with a layer of endothelial cells was calculated according to results of two steps experiment.

Table 6. Adhesion and transmigration of leukocyte cells on endothelial cell layer.

Location	Resultant leukocyte concentration [%]	Permeability
Initial state	14.088	0.388
Final state	5.480	

At the next part, three variant of construct with different gradient of chemoattractant have been prepared, the MSC cells was applied on endothelial layer and their activities during 24 hours was analyzed by confocal microscope. Tab. 7 show scheme of the experiments and cell real cell numbers detected by microscope (measured from area 1,1 × 1,1 mm).

Table 7. Experiment results: c_1, c_2 are concentrations of added to the opposite sides of membrane. chemoattractant (PDGF-beta)

Time [h]	Setup with gradient 0	Setup with gradient 10	Setup with gradient 20
0			
24			
Measured transmigrated cells	0	2	4

Conclusion: the measured transmigration activity is in accordance with theoretical formula (1)

$$\frac{dM}{dt} = \quad (-P).\ \ S.\ \Delta R \tag{1}$$

where M presents cell concentration during the time, t is the time, - P is value of membrane's permeability, S is square of the membrane, ΔR is chemotaxis gradient, can be analyzed with using values (P, S, ΔR) obtained experimental way.

10. Summary

Summing up results of all experiments, is established, that microfluidic model simulated transmigration of immune cells through endothelial wall is described by such parameters like geometric structure (of model and of individual compartments), chemical and physical properties of component materials.

Geometrical parameters of model depend on used way of scanning. In that study, scanning was realized by laser confocal microscope Leica TCS SP8 X. Horizontal planes which can be focused are limited by limits of electronic z-moving of sample holder, in our case z-limit is 1500µm). Geometrical parameters of model (thickness in z axis) must be in this range 1500µm. Geometrical parameters of membrane have been chosen by experimental way, coming from adhesive properties on its surface. PDMS, as basic substance for scaffold have been chosen due to ideal transmittance properties.

Coming from parameters mentioned above, there were offered three microfluidic models. Model 1 (Fig.30) and model 2 (Fig.31) represent different constructional solution with using of the same compartment materials, model 3 (Fig.32) represent combination of advantages of models 1 and 2. That means, model 3 provide good conditions for scanning (because of PDMS transparency), conditions for cell cultivation (because of using Fibronectin) and also represent real capillary system more realistic (because of model form).

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List of used abridgment

2D - two-dimensional

3D - three-dimensional

MSC - mesenchymal stem cell

PDMS - polydimethylsiloxane

DNA - deoxyribonucleic acid

ATP - adenosine triphosphate

UV - ultraviolet

SE – structural element

HUVEC - human umbilical vein endothelial cells

PDGF-beta - platelet-derived growth factor subunit B

Attached files

Files attached to that thesis include commented code for calculation of cell layer concentration, mentioned in Chapter 9.5 and data from confocal microscope, which were used for calculation during experiments.

Data format: Tagged Image File Format (tif) with logical size 512px × 512px, physical size of scanning field 1160 µm × 1160 µm, and z-step 2,5µm. Data names defenition: channel 00 (ch00) – endothelial cells, channel 01 (ch01) – light, channel 02 (ch02) – leukocyte cells.